STUDIES ON THE MECHANISM OF ACTION OF PEDERINE

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ABSTRACT

Pederine, a drug extracted from the coleopter *Paederus fuscipes*, inhibits the growth of in vitro cultured cell lines at concentrations of the order of 1.5 nanogram/ml. Cytological examination shows a generalized cytotoxic effect. Analysis of macromolecular syntheses by the use of radioactive precursors shows that pederine causes an almost immediate block of protein and DNA synthesis, without affecting RNA synthesis. The effects on the synthesis of the two types of macromolecules remain nearly simultaneous even at the lowest active concentrations of pederine. Studies with cell-free systems show that the drug inhibits protein synthesis, whereas it is ineffective on the DNA-polymerizing activity. It seems, therefore, that the drug acts primarily on the amino acid–polymerizing system, and that the effect on DNA is secondary. This idea is strengthened by the observation that puromycin, a specific inhibitor of protein synthesis, also affects promptly DNA synthesis of in vitro cultured cells. Other authors have shown the same phenomenon with a number of inhibitors of protein synthesis; the properties of pederine support, therefore, the view that continuous protein synthesis is necessary for the maintenance of DNA replication in higher organisms.

INTRODUCTION

Pederine[1] is a poisonous substance extracted from the insect *Paederus fuscipes* Curt. (*Coleoptera; Staphylinidae*) and purified up to crystalline state by Pavan and Bo (1). Its chemical structure, shown in Fig. 1, was determined by Cardani et al. (2), and its most relevant biological effects were described by Pavan (3). Previous experiments have shown the remarkable toxic potency of this drug in animals and plants: the LD_{50} for mice, rats, and guinea pigs is of the order of 2 μg per 100 g of body weight; on in vitro cultures of HeLa cells, concentrations of the order of 1 ng/ml (1 nanogram = 10^{-9} g) cause marked inhibition of cellular growth (4); 0.5 μg/ml of pederine causes a strong inhibition of germination of seeds of *Lupinus albus* and produces a metaphasic block on mitoses of root-tip meristems of *Allium cepa* (3). The striking toxicity of this substance prompted a more detailed study of its mechanism of action in order to determine the primary site of attack of the drug. This paper reports a study on the toxicity of the drug on several cell lines cultivated in vitro, a description of the remarkable cytological effects caused by its action, and experiments demonstrating that the substance acts primarily on protein synthesis.

[1] Also called pederin in the literature.

MATERIALS AND METHODS

Reagents

A crystalline preparation of pederine was used throughout the experiments; the drug was dissolved

in water, care being taken that the pH was near neutrality in view of the acid-lability of the drug. Tritiated thymidine (833 μc/μmole) and uridine (500 μc/μmole) were purchased from the Radiochemical Centre, Amersham, England; leucine-^{14}C (83 μc/μmole) and phenylalanine (366 μc/μmole) from the New England Nuclear Corp., Boston; puromycin and mitomycin were products of Nutritional Biochemical Corp., Cleveland, and of Sigma Chemical Co., St. Louis, respectively.

Cell Strains or Lines and Culture Procedures

The minimum inhibitory concentration of pederine was determined both on heteroploid cell lines and on diploid strains. All other experiments were performed with the EUE line only.

Cell Lines

EUE: a human cell line isolated by Terni and Lo Monaco (5).

E6D: an EUE clonal subline deficient in alkaline phosphatase, isolated by De Carli et al. (6).

HeLa: Gey et al. (7).

Hep 2: Fjelde (8).

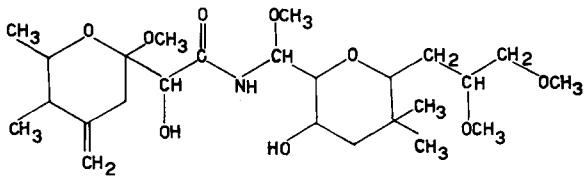

FIGURE 1 The structure of pederine: after Cardani et al. (2).

AS: a cell line isolated from the subcutaneous tissue of a patient with trisomy 21, and maintained in our laboratory for 4 yr.

MEF: a cell line isolated in 1964 from a mouse embryo by Dr. Murthy at the Research Laboratories of the Lepetit Corporation, Milan, Italy.

37 RC: a pseudodiploid cell line isolated from the kidney tissue of a *Cercopithecus* (Nuzzo et al. 9).

KB: Eagle (10).

BHK 21: Stoker and MacPherson (11).

Cell Strains

Z 1: a diploid cell strain derived from human thyroid, grown in our laboratory for 5 months.

M 1: a diploid cell strain derived from human amnion, grown in our laboratory for 6 months.

The stock cultures of all the cell lines (except BHK 21 and MEF) were grown in Lactalbumin medium (Hanks' BSS, 5 mg/ml of Lactalbumin hydrolyzate, 50 μg/ml of yeast extract); BHK 21, MEF, and the diploid strains were grown in Eagle's medium (12). Both media were supplemented with 10% of calf serum. In all the experiments, the cells were grown with Eagle's medium at 37°C.

Toxicity Tests

Each cell line or strain was exposed to concentrations of pederine ranging, by twofold dilution, between 100 and 0.3 ng/ml. The cells were inoculated into 5-ml screw-cap vials (A. Thomas Co., Philadelphia) containing 2 ml of Eagle's medium. The inoculum size was 5×10^4 cells per vial. The cells adherent to the bottom of the vials were incubated at 37°C for 4 days, in parallel with controls without pederine. After this period, the lowest concentration of pederine causing total inhibition of cellular growth was recorded.

Cytological Examinations

Cells grown on the bottom of 5-ml screw-cap vials were detached with a 2.5-mg/ml solution of trypsin (Difco, Detroit, 1:250). After hypotonic treatment with an 1% solution of sodium citrate, and prefixation with a few drops of 3:1 (v/v) methyl alcohol-acetic acid solution, the cells were fixed with the same reagent for 10 min; they were thereafter spread on slides previously wetted with 0.1% Haemosol solution (Dade Reagents Inc., Miami). The slides were air dried, stained with Giemsa solution (E. Merck Ag., Darmstadt, Germany), and mounted with Euparal (Chroma Gesellschaft, Stuttgart, Germany).

Macromolecular Syntheses

The syntheses of DNA, RNA, and protein were followed by the incorporation into acid-insoluble material of radioactive thymidine, uridine, and L-leucine, respectively. The cells were grown on the bottom of 5-ml screw-cap glass vials; each vial corresponded to a single time-point. 16 hr before the addition of the radioactive material, approximately 1.5×10^5 cells were inoculated in a number of vials containing 2 ml of Eagle's medium. When the cells numbered approximately 2.5×10^5, the radioactive precursors and the inhibitors were added simultaneously (within 1 min) in all the appropriate tubes; at the indicated times, the tube corresponding to a given time-point was emptied of medium, the cells were washed with 2 ml of ice-cold saline, and 1 ml of a 0.5 mg/ml solution of sodium

dodecyl sulfate (Sigma Chemical Co.) was added to the tube; the cells were lysed by incubation at 37°C for 10 min. The lysate was then homogenized by pipetting it vigorously five times through a narrow-bore 2-ml pipette; after the addition of 1 ml of ice-cold 7% perchloric acid, the tube was kept at 0°C for 10 min; its content was then poured onto a paper filter (Schleicher-Schüll "Selecta," No. 602-h) having a diameter of 2.4 cm; the filter was washed five times with 5-ml aliquots of cold 7% perchloric acid, washed with 1:1 (v/v) alcohol-ether, then washed with ether, and finally dried; the radioactivity was counted on a Packard Liquid Scintillation Counter, Model 4322.

When DNA synthesis was determined by a chemical test, the cells were incubated as above; at the indicated times, they were washed with cold saline, homogenized by the addition of 1 ml of 0.2 N NaOH, and subsequently incubated at 37°C for 10 min; the macromolecules were then precipitated by the addition of 1 ml of cold 7% perchloric acid, and after 10 min at 0°C they were centrifuged at 10,000 *g* for 10 min. The pellet was assayed for DNA according to Burton (13).

RESULTS

Toxicity Tests

The lowest concentration of pederine causing total inhibition of growth of in vitro cultured cells after 4 days was determined on a number of cell lines. Table I shows that a concentration of approximately 1.5 ng/ml is sufficient to block cell growth. A more sensitive study was carried out on the EUE line by determining the effect of decreasing concentrations of pederine on the plating efficiency (i.e. on the number of macroscopic colonies visible after 2 wk divided by the number of cells plated). A clear-cut end point of inhibition was found: at 0.3 ng/ml no colonies were observed, whereas at 0.1 ng/ml the efficiency of plating and the average colony size were the same as in the control.

TABLE I

Minimum Inhibitory Concentrations (M.I.C.) of Pederine on Different Strains and Cell Lines

Strain or line	M.I.C ng/ml
EUE	1.5
E6D	1.5
HeLa	1.5
KB	1.5
Hep	1.5
AS	1.5
MEF	1.5
CE	1.5
BHK	1.0
Z 1	3.1
M 1	3.9

For the description of the lines and strains, and for the procedure, see Materials and Methods.

Cytological Observations

The block of dividing ability of cells was associated with marked cytological modifications. In order to follow the progression of the cytopathogenic effect we treated the cell cultures with 100 ng/ml of pederine for periods ranging from 0–20 hr. After 50 min, the first visible effect was a marked decrease in the frequency of mitoses; longer treatments produced evident signs of cellular degeneration, such as metaphase-blocked mitoses with abnormally-looking chromosomes; the latter were often clustered in small groups. In resting cells, the cytoplasm became more refringent and showed a large number of vacuoles. After 5–20 hr of treatment, nuclear fragmentations were observed, followed by total cellular lysis.

Stained preparations showed basophilic areas, probably due to the release of nuclear material into the cytoplasm. This phenomenon is clearly seen in Fig. 2.

Macromolecular Syntheses

The profound alterations observed at the morphological level suggest the impairment of processes or structures of crucial importance for the cell; it was thought that the primary site of action of the drug could be determined by a study of its effect on the basic cellular functions, such as the synthesis of macromolecules; if, at very early times following addition of the drug, and before any morphological alterations were evident, one of the basic syntheses were affected, it could be tentatively considered as the primary target of pederine.

PROTEIN AND DNA SYNTHESIS: The synthesis of DNA was investigated by measuring the incorporation of radioactive thymidine into acid-insoluble material. As shown in Fig. 3, within 10 min after the addition of 100 ng/ml of pederine, DNA replication rate is reduced to less than $\frac{1}{10}$ of that of the untreated control; in the same experiment, protein synthesis, also measured by the incorporation of a radioactive precursors, is blocked even more promptly (see Fig. 3 *b*) and

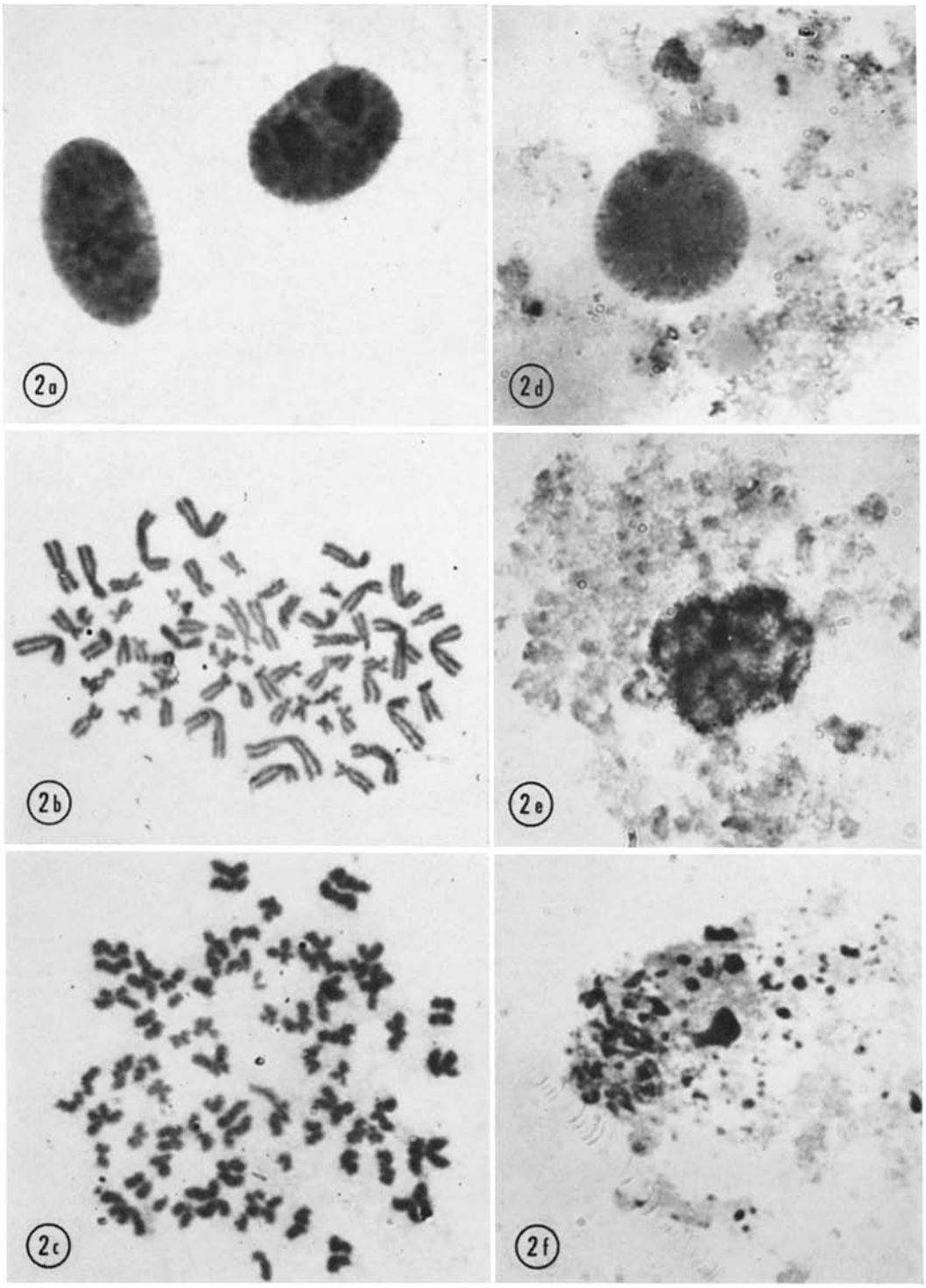

FIGURE 2 Progression of the cytotoxic effect with 100 ng/ml of pederine. All preparations were preceded by hypotonic treatment, and the staining method was specific for nuclei. Under these conditions, the cytoplasm is normally not visible. *a*. Nuclei from normal cells grown in the absence of pederine. *b*. Normal metaphase in a control culture. No colchicine added. *c*. A metaphase after 90 min of exposure to pederine. The chromosomes are highly condensed and their chromatidic components tend to fall apart. *d*. A cell after 3 hr of exposure to pederine. Some highly stainable material is visible in the cytoplasm. *e*. A cell after 5 hr of exposure to pederine. The nucleus shows signs of degeneration, i.e. irregular edges and large masses of pycnotic material. *f*. A cell after 22 hr of exposure to pederine. The nucleus is fragmented and the cell is undergoing a complete lysis.

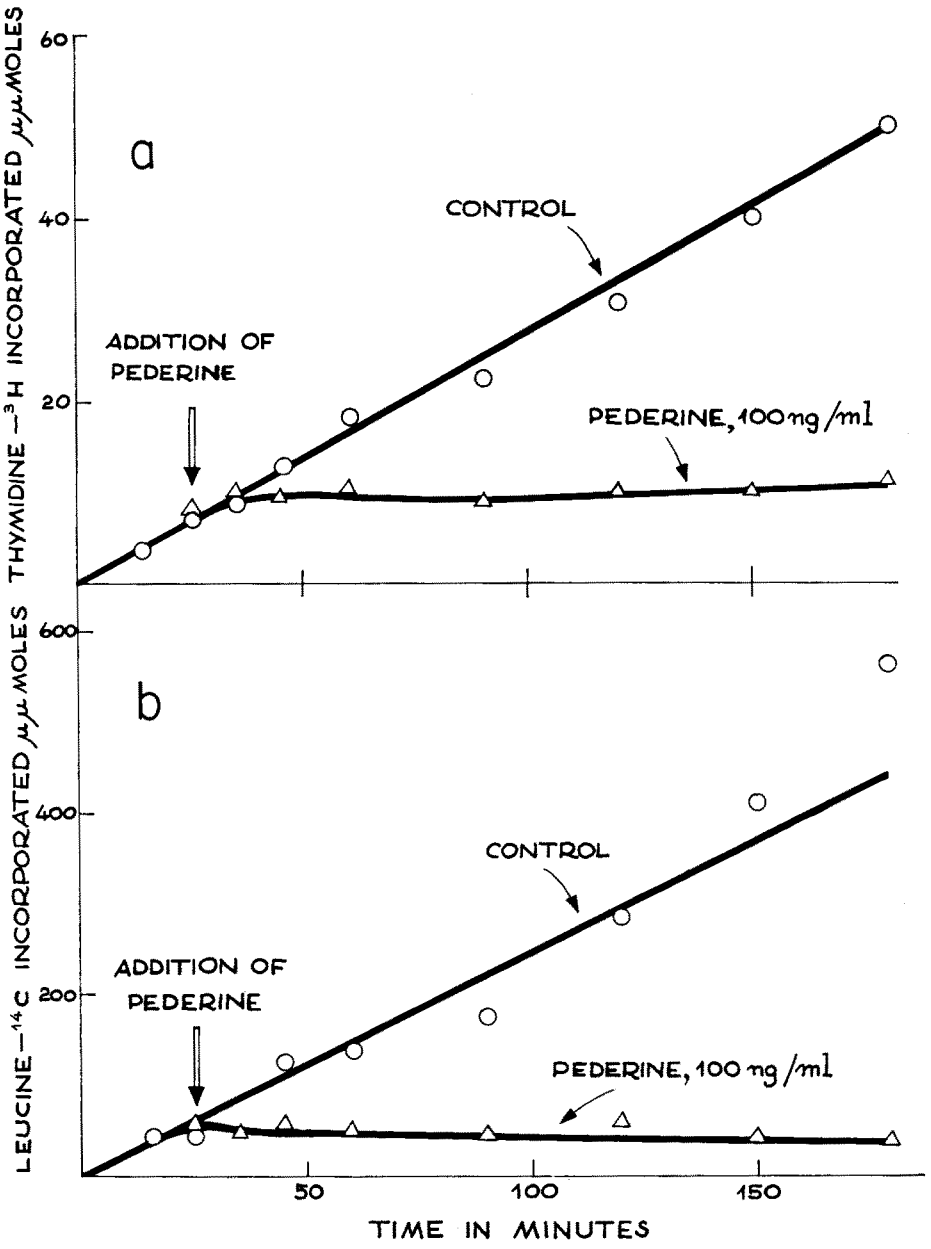

FIGURE 3 Effect of pederine on DNA and protein synthesis of in vitro cultured cells. Each vial contained 20 μc of thymidine-^{3}H and 2 μc of leucine-^{14}C. Where indicated, pederine was added to a final concentration of 100 ng/ml, 25 min after addition of the radioactive material; for the procedure, see Materials and Methods. *a*, DNA synthesis; *b*, protein synthesis.

perhaps more completely, as suggested by the slightly negative slope of the incorporation curve following addition of the drug.

LACK OF EFFECT ON RNA SYNTHESIS: The essentially simultaneous block of the two syntheses could be the consequence of a more generalized cell damage influencing all cellular processes; the experiment reported in Fig. 4 shows that this is not the case: 100 ng/ml of pederine have no appreciable effect on RNA synthesis; the

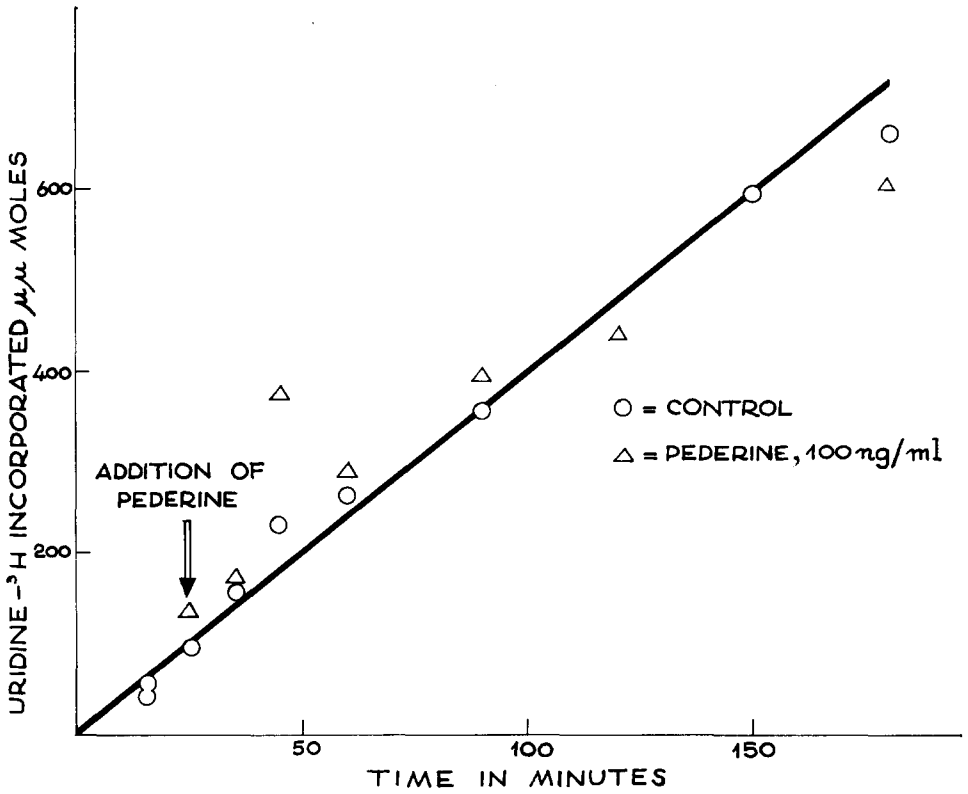

FIGURE 4 Effect of pederine on RNA synthesis. Each vial contained 10 μc of thymidine-^{3}H; other details as in Fig. 3.

lower value observed after 180 min cannot be considered significant, in view of a certain scattering of the other points in this experiment.

EFFECT OF LOWER CONCENTRATIONS: In order to determine which of the two syntheses was blocked first, we progressively lowered the concentration of inhibitor so that conditions might be found under which one of the two processes would be affected before the other one. Fig. 5 shows that even at concentrations as low as 1.5 ng/ml no clear-cut difference could be observed either in the timing or in the extent of decrease of the rate of synthesis for the two macromolecules.

RECOVERY FOLLOWING REMOVAL OF THE DRUG: The same question was approached by determining the time of recovery of the processes in question after a 40-min treatment with 10 ng/ml of pederine and its subsequent removal. As shown in Fig. 6, protein synthesis seems to begin recovering between 2 and 4 hr following drug removal, whereas DNA synthesis rises again only between 4 and 8 hr after. This finding could indicate that protein synthesis is the first process affected by the drug; the recovery of protein synthesis would then be essential for the restoration of other processes, including DNA synthesis. In fact, in bacterial systems the removal of chloramphenicol yields similar results (14), but our data are undoubtedly not so clear-cut as hoped, and the interpretation given is not immune to criticisms.

ACTION OF PUROMYCIN AND MITOMYCIN: An answer to the question could be obtained by comparing the effect of pederine with that of other drugs the mechanism of action of which is well established. The drug chosen was puromycin; its structure and detailed mechanism of action were described by Nathans (15), who showed that this substance mimics the terminal 3′ end of an aminoacyl-sRNA and is thus incorporated terminally into a growing polypeptide chain and prevents any further synthesis. Fig. 7 shows the effect of 25 μg/ml of puromycin on protein and DNA synthesis in our cell strain: here too, the

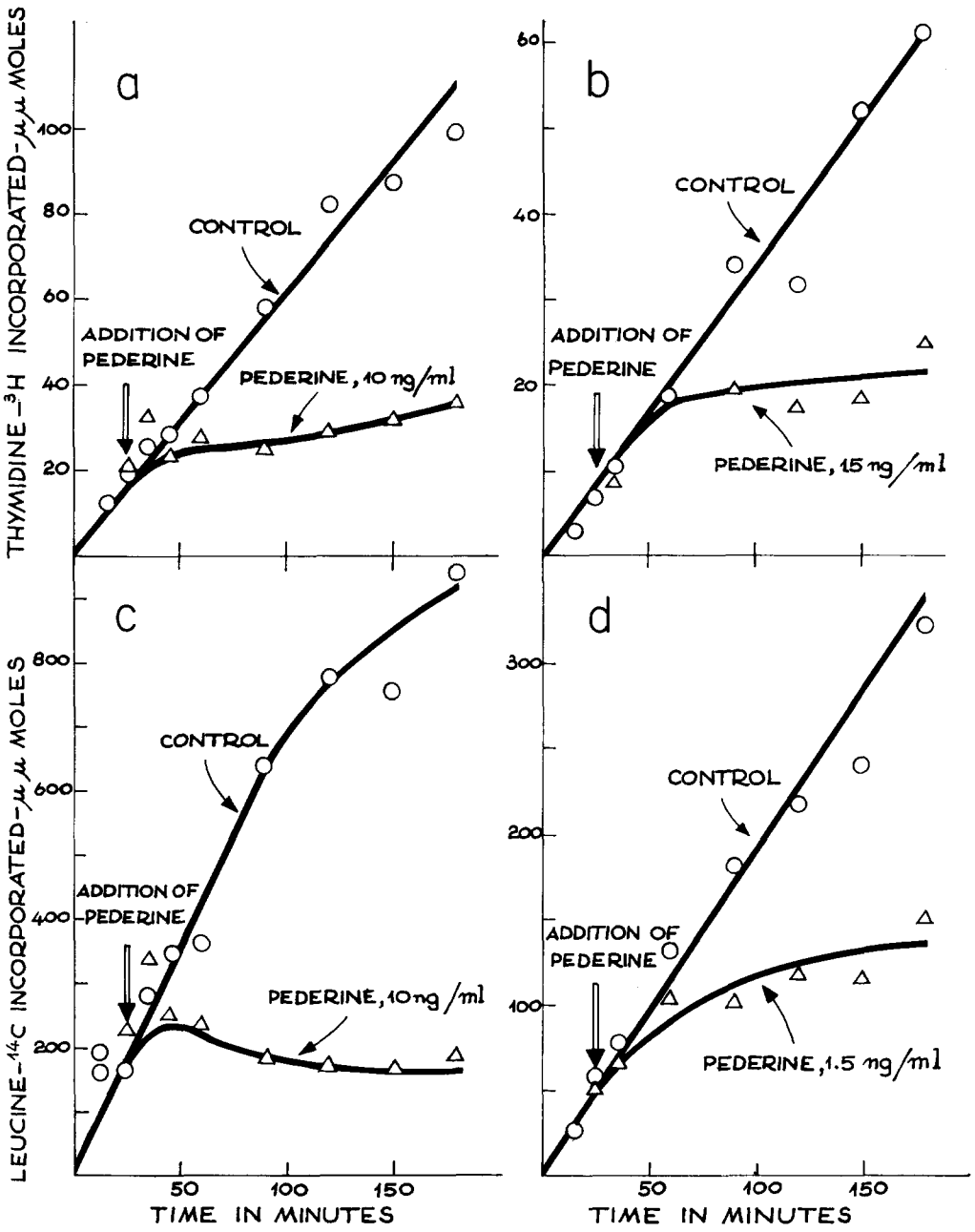

FIGURE 5 Effect of decreasing concentrations of pederine on DNA and protein synthesis. Each vial contained 20 μc of thymidine-^{3}H and 2 μc of leucine-^{14}C. 25 min after the addition of the precursors, pederine was added to the appropriate tubes at final concentrations of 10 ng/ml (*a* and *c*) or 1.5 ng/ml (*b* and *d*); for the procedure, see Materials and Methods. *a* and *b*: DNA synthesis; *c* and *d*: protein synthesis.

block of protein synthesis is essentially simultaneous with a profound impairment of thymidine incorporation. In fact, the curves of Fig. 7 are essentially indistinguishable from those of Fig. 3.

The simultaneous block of protein and DNA synthesis seems, at first sight, rather puzzling. In fact, data from bacterial systems lead one to expect that, if DNA synthesis is affected first, an effect on

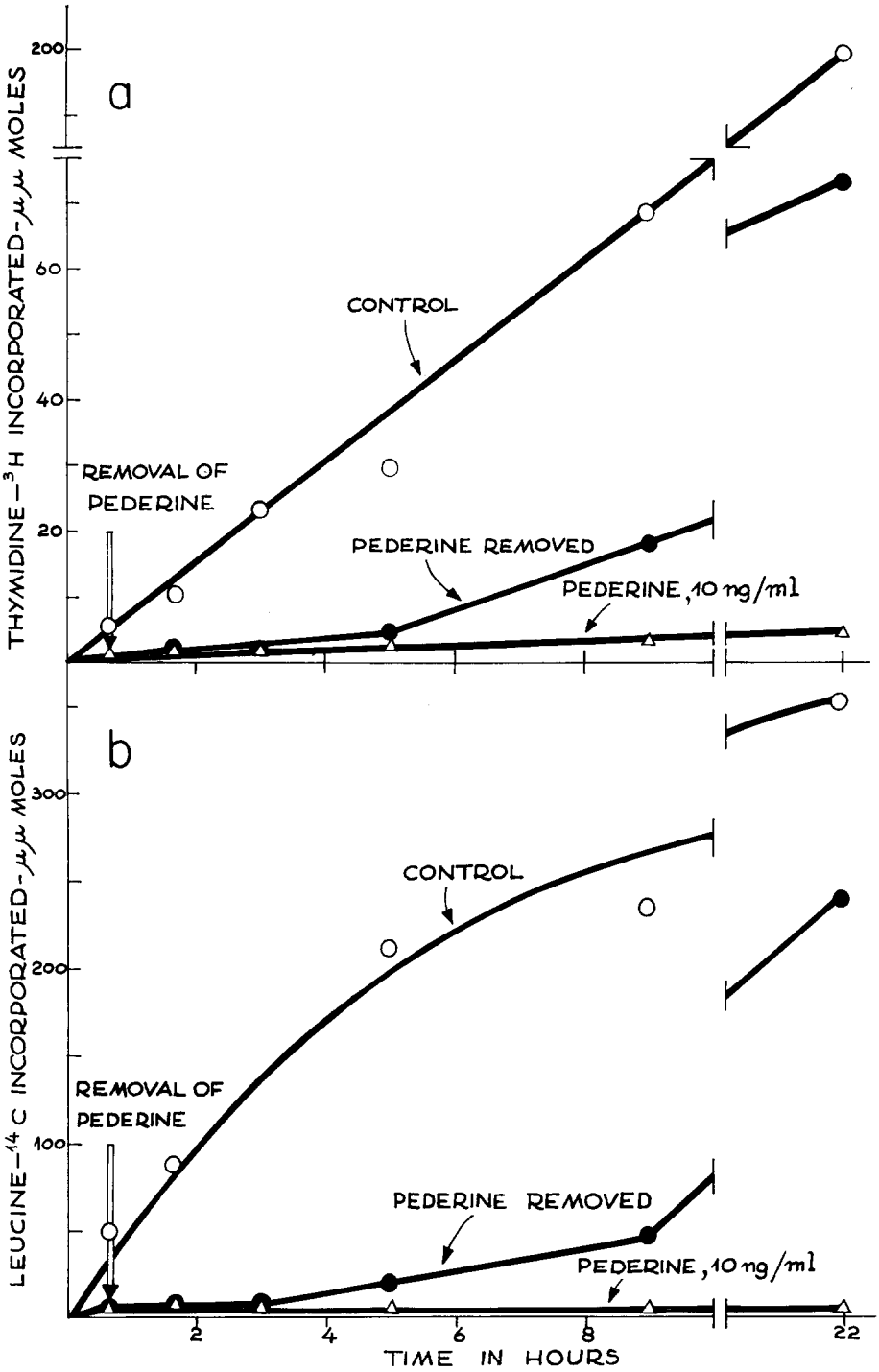

FIGURE 6 Recovery of DNA and protein synthesis after removal of pederine. To each vial were added, at zero time, 5 μc of thymidine-^{3}H and 0.25 μc of leucine-^{14}C; at the same time, pederine was added to the indicated tubes at a final concentration of 10 ng/ml; 40 min later, the medium was poured out of a portion of the pederine-containing vials and the cells were washed twice with 2 ml aliquots of medium; 2 ml of medium containing the radioactive material were then added, and the incubation was continued. *a*, DNA synthesis; *b*, protein synthesis.

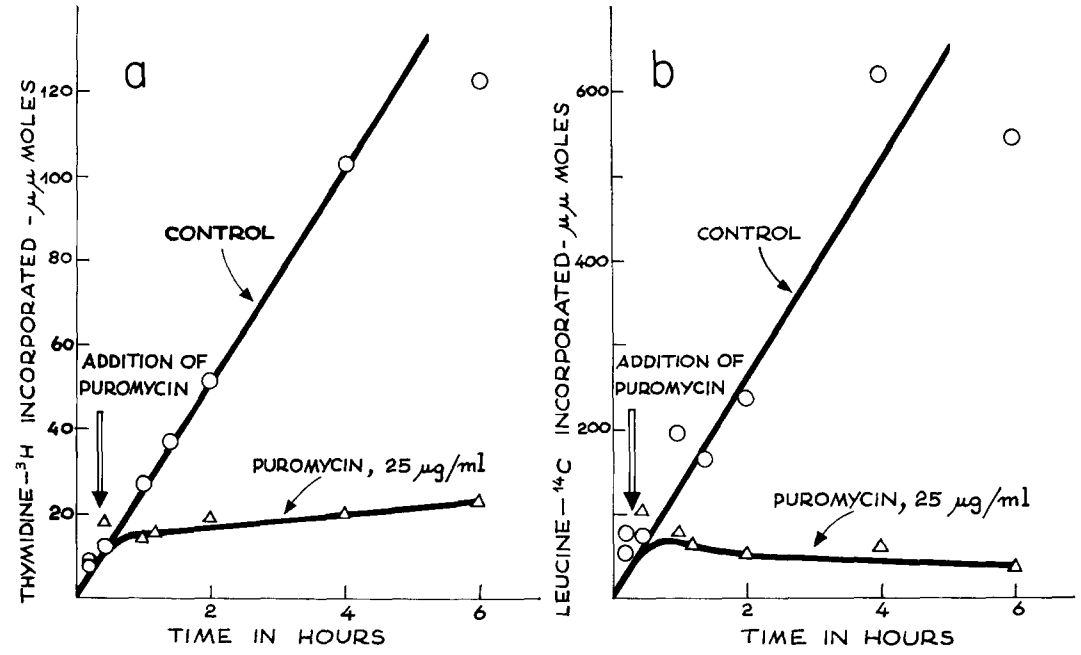

FIGURE 7 Effect of puromycin on DNA and protein synthesis. Each vial contained 20 μc of thymidine-^{3}H and 2 μc of leucine-^{14}C; to the indicated vials, puromycin was added at a final concentration of 25 μg/ml, 25 min after the radioactive material. *a*, DNA synthesis; *b*, protein synthesis.

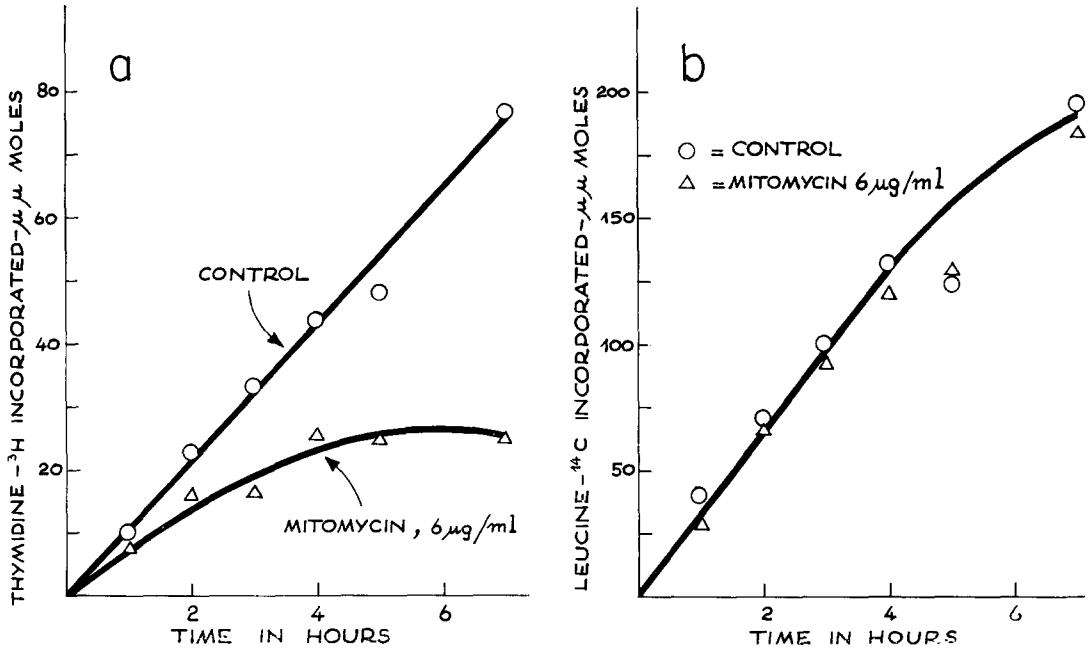

FIGURE 8 Effect of mitomycin on DNA and protein synthesis. Each vial contained 5 μc of thymidine-^{3}H and 0.25 μc of leucine-^{14}C; at zero time, mitomycin was added to the indicated tubes at a final concentration of 6 μg/ml. *a*, DNA synthesis; *b*, protein synthesis.

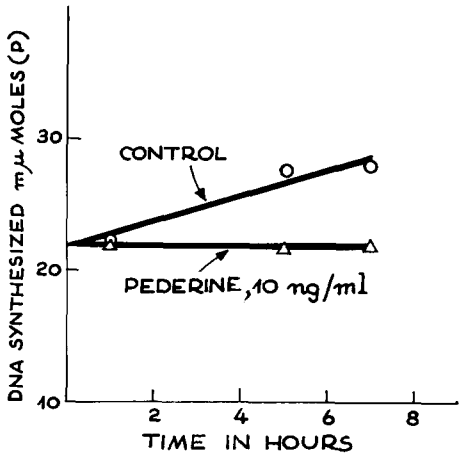

FIGURE 9 Effect of pederine on DNA synthesis as measured by chemical tests. The indicated tubes contained 10 ng/ml of pederine. For the procedure, see Materials and Methods. Each point is an average of five determinations on parallel samples.

protein synthesis should be apparent only much later, and likely be preceded by the arrest of RNA synthesis; one would actually expect that protein synthesis should continue until all messenger RNA is used up and transcription of the genome impaired. Conversely a block of protein synthesis affects DNA synthesis only after the chromosome has completed its replication (16).

It is to be noted that, as expected, the block of DNA synthesis by another agent, whose mechanism of action is well known, does not entail any early impairment of protein synthesis in our system; the mechanism of action of mitomycin was elucidated by Szybalski (17) and shown to consist in the formation of cross-links between the two helices of DNA, causing an immediate and specific block of DNA synthesis. In our cells, as shown by Fig. 8, mitomycin affects DNA replication, leaving protein synthesis unaltered. It is thus evident that the apparent coupling of protein synthesis and DNA synthesis does not go in both directions; it would seem rather that continuous protein synthesis is necessary for DNA replication to occur.

CHEMICAL DETERMINATION OF DNA SYNTHESIS: It was important for us to ascertain that the observed phenomenon was not an artefact due to the particular technique used to measure DNA synthesis, namely, incorporation of labeled thymidine. In fact, it is conceivable that an alteration of protein synthesis might interfere with the level of thymidine kinase and result in a lack of incorporation of this nucleoside, whilst DNA synthesis would go on as normal by the thymidylate synthetase pathway. This possibility was

TABLE II

Effect of Pederine on Cell-Free Protein Synthesis

Additions	Phenylalanine-^{14}C incorporated
	μμmoles
None	10.3
Pederine, 10 μg/ml	1.2
Pederine, 1 μg/ml	5.7
Pederine, 0.1 μg/ml	7.9
Puromycin, 400 μg/ml	3.4

The assay was performed in a final volume of 0.25 ml. Each tube contained 0.075 mμmoles of phenylalanine-^{14}C, ribosomes corresponding to 45 μg of protein, 0.32 mg of supernatant protein, and 40 μg of polyuridylate. The preparation of the amino acid incorporation system from EUE cells and the assay procedure will be described elsewhere.[2]

TABLE III

Effect of Pederine on Cell-Free DNA Synthesis

Additions or omissions	dAMP-^{3}H incorporated
	μμmoles
Complete system	86
Omit cell extract	<2
Omit denatured DNA	5
Omit dGTP, dCTP, dTTP	6
Add pederine, 6 μg/ml	86
Add pederine, 10 μg/ml	84

The complete system contained, in a final volume of 0.25 ml, 25 mμmoles of dATP-^{3}H (Schwarz Bioresearch, Orangeburg, N.J.), 25 mμmoles each of dGTP, dCTP, dTTP (Sigma Chemical Co., St. Louis), 2 μmoles of $MgCl_2$, 2μmoles of β-mercaptoethanol, 25 μmoles of Tris-HCl buffer, pH 7.5, 10 μg of denatured DNA, and 200 μg of crude extract from EUE cells; the preparation of the extract and the procedure for the assay were as described by Gold and Helleiner (24). The control tube having the complete system was in the linear portion of the assay.

checked by assaying chemically the replication of DNA in the presence of pederine: as shown in Fig. 9, the drug causes a real and immediate block of the formation of new DNA by the cells.

ACTION OF PEDERINE ON CELL-FREE EXTRACTS: The most direct proof of the site of action of pederine was obtained by studying its effect on cell-free systems for the synthesis of protein or of DNA; an amino acid incorporation system from EUE cells has been developed by Perani et al.[2] As shown in Table II, the synthesis of polyphenylalanine stimulated in this system by polyuridylate was inhibited by pederine concentrations 100 times lower than those of puromycin giving a comparable effect.

Conversely (Table III), the activity of DNA polymerase in crude extracts of the same cells is unaffected by as much as 10 μg/ml of pederine.

DISCUSSION

The most outstanding feature of pederine is undoubtedly its potency: concentrations of the order of 1.5 ng/ml, corresponding to 3×10^{-9} M, are sufficient to cause cellular death within 4 days in all the cell lines analyzed, and an immediate impairment of protein and DNA synthesis; this amount of drug corresponds to a maximum of approximately 10^7 molecules available per cell, if one assumes that the cultures concentrate immediately all the drug within the cells. Pederine is, therefore, from 1,000 to 10,000 times more active than the most common antimetabolites. The cytological observations on the effect of pederine are of little help for the study of the specificity of action of the compound: in fact, all the cell alterations observed are suggestive of a generalized cytopathogenic effect. The analysis of macromolecular syntheses seems to yield more information.

The block of protein synthesis seems to take place immediately, i.e. within 10 min after the addition of the drug, and, therefore, much earlier than any appreciable morphological alterations; the block of DNA replication is nearly simultaneous with that of protein synthesis, but is perhaps of a slightly less degree. This little difference (Fig. 2), and the earlier recovery of amino acid incorporation after removal of the drug (Fig. 5), indicate that protein synthesis is affected first; this conclusion is strengthened by the apparent identity of the effects of pederine and puromycin (Fig. 6), and by the opposite behavior of mitomycin. More direct proof is given by the data with cell-free extracts: pederine inhibits markedly cell-free synthesis of proteins much more than puromycin does, whereas it does not appreciably affect the enzymatic synthesis of DNA.

The apparent tight coupling of protein synthesis and DNA synthesis while RNA synthesis is unaffected remains to be explained. In fact, this phenomenon has already been described in higher organisms (18, 19), and recently data very similar to ours have been obtained by Young (20) who used puromycin and a number of other agents specific for blocking protein synthesis: all these substances, when assayed on in vitro cultured cells, reduce markedly within a few minutes also the rate of DNA replication, without affecting RNA synthesis.

As mentioned above, this finding is not in agreement with what happens in bacterial systems, where only the *initiation* of chromosomal replication is dependent on protein synthesis, and DNA replication stops, on the average, approximately one generation time after the block of protein synthesis. In our system, instead, where the generation time is of about 24 hr, and the completion of chromosome replication takes at least 6 hr, DNA synthesis halts within a few minutes following the arrest of protein synthesis.

Other authors have already discussed the possible causes of this tight coupling (18–20). We shall briefly mention the most obvious hypotheses:

1. A postulated need for either continuous histone synthesis or production of a hypothetical lipoprotein fraction;
2. The synthesis of protein linkers;
3. The subdivision of the eukaryote chromosome into a number of functional subunits (possibly corresponding to the "replicons" described by Jacob et al., 21), each requiring the synthesis of its "initiator" protein(s) for the starting of replication; in this case, if the number of subunits were large enough, the time-lag between the block in protein synthesis and the block in DNA replication could be of the order of minutes, as observed in the experiments described here.

Recent data of Cairns (22) and of Plaut et al. (23) are in agreement with the postulates of hypothesis 3, showing at least 100 replication subunits per human chromosome in one case, and 50

[2] Perani, A., B. Parisi, and O. Ciferri. Manuscript in preparation.

per *Drosophila* chromosome in the other. Such a high number of functional subunits could account for our results.

The data presented here have demonstrated that pederine blocks protein synthesis in mammalian cells. The remarkable potency of the drug stimulates interest in a knowledge of its detailed mechanism of action. This drug could become then an agent of choice for a study to determine the reasons for the strict requirement for continuous protein synthesis in order to maintain the normal replication rate of eukaryote chromosomes. The understanding of this phenomenon could probably help to clarify the structural and functional organization of the chromosomes of higher organisms.

This work was supported by a grant from Euratom-CNR-CNEN, Contract No. 012-61-12 BIAI. One of us (M. P.) was supported by a grant from CNR, Rome.

Received for publication 14 August 1967, and in revised form 6 November 1967.

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